

# An antimicrobial effect associated with rabbit primary aqueous humour

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## Abstract

Viability curves were recorded for a variety of bacteria suspended in rabbit primary aqueous humour. Controls included neat rabbit serum, dilute rabbit serum (1/50), dilute heat treated fetal bovine serum (1/50), and balanced salt solution. Rabbit aqueous humour was bactericidal in vitro for *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *Micrococcus* spp, *Streptococcus pneumoniae* and *Escherichia coli* were not affected. The factor mediating the bactericidal effect survives filtration (0.2 µm porosity) and freezing in liquid nitrogen (-196°C) but is labile when stored in air at temperatures of 4°C, 25°C, and 37°C for 1 hour. The bactericidal effect was stable when aqueous humour was stored in an atmosphere of 5% CO<sub>2</sub> at 37°C for 3 hours. The antibacterial component in rabbit aqueous humour has yet to be identified.

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Cataract surgery has a low postoperative infection rate. Published incidences range from 0.022% to 1.42%,<sup>1,2</sup> averaging 0.67% - well below the 7.5% incidence seen in other surgical sciences.<sup>3</sup>

These figures have been thought to confirm the superiority of ophthalmic antimicrobial surgical technique.<sup>4</sup> This proposal is countered, however, by Vafidis<sup>5</sup> who demonstrated bacterial contamination of 26% of intraocular lenses inserted into 50 eyes during extracapsular cataract extraction (ECCE), and by Sherwood<sup>6</sup> who demonstrated contamination of 29% of anterior chamber aspirates taken from 101 eyes undergoing ECCE. Dickey<sup>7</sup> took aspirates from the anterior chambers of 30 patients undergoing uncomplicated ECCE and found 43% to be culture positive. That none of these eyes developed endophthalmitis was considered due to the inoculum being of small size and of low virulence.

In vivo animal work, however, suggests that the eye has innate resistance to bacterial infection. Meredith<sup>8</sup> demonstrated rapid sterilisation 3 days after intravitreal injection of between 5000 and 10 000 *Staphylococcus epidermidis* cells into rabbit eyes (which had previously undergone lens removal), while Davey<sup>9</sup> compared the growth of *Klebsiella pneumoniae* in the vitreous cavity of normal rabbit eyes with that in subcutaneous croton oil pouches (artificial oil reservoirs). The vitreous exhibited rapid sterilisation, while the organisms within the oil pouches continued to multiply over a 5 day period.

Beyer<sup>10</sup> inoculated *Staphylococcus aureus* into the anterior chamber of monkey eyes following

lens extraction. Where the posterior lens capsule formed an intact barrier between aqueous and vitreous, 10 000 organisms were eradicated. In the absence of this barrier an endophthalmitis occurred following a much smaller infectious load. Shockley *et al*<sup>11</sup> performed a similar experiment using rabbit eyes and *Pseudomonas aeruginosa*: where the posterior lens capsule was intact the anterior chamber could eradicate an infectious load 20 times greater than that which induced endophthalmitis when inoculated into the vitreous.

Maylath and Leopold<sup>12</sup> inoculated *Staphylococcus aureus* into normal rabbit eyes: after introducing 700 organisms into the vitreous cavity endophthalmitis rapidly ensued, while the corneal stroma eradicated this number; in contrast, the anterior chamber could eradicate 5000 organisms. He concluded that the eye possessed an effective antimicrobial defence that was most efficient in the anterior chamber. The high ratio of culture positive vitreous to aqueous taps in known cases of endophthalmitis<sup>10 13 14</sup> may be a reflection of these findings.

Various hypotheses could explain an innate protection against bacterial infection possessed by the anterior chamber: (1) that aqueous humour is nutritionally inadequate to support the growth of bacterial organisms; (2) that invading micro-organisms are eluted from the anterior chamber with the bulk flow of aqueous humour via the trabecular meshwork; (3) that aqueous humour contains significant amounts of lysozyme, lactoferrin, transferrin, complement, immunoglobulin, opsonins, or other, as yet unrecognised antibacterial factor(s); and (4) a combination of some, or all of these factors.

This project has been designed to investigate the presence and nature of antibacterial factors within rabbit primary aqueous humour.

## Materials and methods

### AQUEOUS HUMOUR

Aqueous humour was collected from adult New Zealand White rabbits of both sexes, weighing between 2 and 2.5 kg. The rabbits were bred for domestic consumption and slaughtered by cutting the major vessels in the neck. A volume of 50-75 µl of aqueous humour was collected immediately after death via a 27 gauge needle inserted at the corneal limbus, snap frozen in liquid nitrogen and stored at -70°C. Before each experiment, individual samples of aqueous humour were thawed and pooled.

Aqueous humour was collected from batches of approximately 70 animals exposed to similar conditions before slaughter. A small amount of aqueous humour was collected from rabbits bred

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for research purposes under controlled conditions.

#### ORGANISMS

The organisms used in this study included laboratory strains of *Staphylococcus aureus*, National Collection of Type Culture (NCTC) number 6571, *Pseudomonas aeruginosa* (NCTC 10662), *Escherichia coli* (NCTC 10418), *Staphylococcus epidermidis* (NCTC 4276), and clinical isolates of *Streptococcus pneumoniae* and *Micrococcus* spp.

#### BACTERIAL PREPARATION

A volume of 100 µl of an overnight broth was inoculated into 10 ml of brain/heart infusion broth (Oxoid, Basingstoke, UK) and incubated at 37°C. After 2 hours the organisms were spun, washed twice in phosphate buffered saline (PBS, Sigma Chemical Co, Poole, UK), re-spun, and suspended in PBS to give a final count of approximately 10<sup>6</sup> colony forming units/ml (CFU/ml).

#### BACTERIAL KILLING CURVES

A volume of 100 µl of bacterial suspension was inoculated into 900 µl of rabbit aqueous humour and incubated at 37°C in 12 mm diameter glass test tubes with loose fitting caps. At intervals, 100 µl of culture were removed and serially diluted into PBS. Viable bacterial counts were recorded on DST agar (Oxoid, Basingstoke, UK) using either the Spiral System (Spiral Systems, Cincinnati, OH) or the method of Miles *et al.*<sup>15</sup> Control killing curves were obtained by simultaneous inoculation of organisms into balanced salt solution (BSS, Alcon Laboratories, Watford, UK) whole rabbit serum (Sigma Chemical Co, Poole, UK), dilute rabbit serum (1/50 rabbit serum/BSS), and heat treated, dilute fetal bovine serum (56°C for 30 minutes of 1/50 fetal bovine serum/BSS) (Sigma Chemical Co, Poole, UK).

#### SIZE OF BACTERIAL AGGREGATES

Standard killing curves were initiated as above using filtered (Acrodisc 0.2 µm low protein binding filter, Gelman Sciences, Ann Arbor, MI, USA) rabbit aqueous humour, and BSS. At intervals, 1 ml cultures were fixed for 1 hour using 20 µl of 20% glutaraldehyde in aqueous solution (Sigma Chemical Co, Poole, UK). Cultures were then mixed with 25 ml of filtered (0.22 µm) PBS and particle size recorded using a 50 µm aperture on a Coulter Counter ZM (Coulter Electronics Ltd, Luton, UK).

#### LONGEVITY OF THE BACTERICIDAL EFFECT

Standard killing curves were initiated as above. After 3 hours the aqueous humour/bacterial suspension was spun and the organisms resuspended in freshly thawed aqueous humour.

#### PRETREATMENT OF AQUEOUS HUMOUR

Thawed, pooled aliquots of rabbit aqueous

humour were subjected to a variety of pretreatments to investigate the stability of the antibacterial effect.

#### Effect of refreezing

The aqueous humour was snap frozen for a second time in liquid nitrogen (−196°C) before bacterial inoculation.

#### Effect of temperature

The aqueous humour was stored in air at different temperatures for 30–60 minutes before bacterial inoculation.

#### Effect of filtration

The aqueous humour was passed through 0.2 µm filters (Acrodisc 0.2 µm low protein binding filter, Gelman Science, Ann Arbor, MI, USA and 0.2 µm Minisart, Sartorius GmbH, Gottingen, German) before inoculation.

#### Effect of dilution

The aqueous humour was serially diluted with balanced salt solution before inoculation.

#### Effect of an atmosphere of 5% CO<sub>2</sub>

The aqueous humour was exposed to an atmosphere of 5% CO<sub>2</sub> at 37°C for 3 hours before inoculation.

#### AQUEOUS HUMOUR ANALYSIS

Aqueous humour proteins were separated by electrophoresis in 1.5 mm thick, 10% polyacrylamide gels in the presence of sodium dodecyl sulphate (SDS-PAGE) in a 'Mini-protein II' cell (Bio-rad Laboratories Ltd, Hemel Hempstead). Specimens containing approximately 0.02 mg protein were mixed (1:1) with Laemmli's sample buffer<sup>16</sup> and run under reducing conditions. Commercial molecular weight standards (Sigma Chemical Co, Poole, UK) were used. SDS-PAGE gels were fixed and stained in 0.25% Coomassie blue, 40% methanol, 10% acetic acid for 1 hour, then destained in three changes of 40% methanol, 10% acetic acid.

Total aqueous humour protein was measured using the method described by Johnson and Lott.<sup>17</sup> The electrolytic profile of aqueous humour was analysed using the Hitachi 717 random access analyser (Hitachi, Boehringer Mannheim, Germany).

The polymorphonuclear cell content of the aqueous humour was analysed using light microscopy after centrifugation of 2 ml at 2000 rpm for 10 minutes.

#### STATISTICAL ANALYSIS

Statistical analyses were carried out on the logarithms of the viable counts. When replicate determinations were available, a straightforward two way analysis of variance allowed direct determination of experimental error. In these instances, orthogonal comparisons between

treatments were made by means of Fisher's F or Student's *t* tests.

For experiments without replicate determinations at each sampling point (for example, the *Pseudomonas aeruginosa* data), the following method was used. A regression analysis of variance was performed on the linear (growth) region of the graph. The residual variance extracted from this analysis was then used as an estimate of average experimental error which was applied to each sampling point. Scheffé's statistic was used to determine statistical significance.

## Results

### ANTIBACTERIAL ACTIVITY

There was a reduction in viable colony count for *Staphylococcus aureus* and *Pseudomonas aeruginosa* cultured in rabbit aqueous humour compared with the controls (Fig 1A, 1B, and 1C). The reduction in colony numbers (CFU/ml) of *Staphylococcus aureus* was significant between 60 and 90 minutes ( $p < 0.001$ , analysis of variance, single tailed Student's *t* test,  $df=4$ ). For *Pseudomonas aeruginosa* the difference was

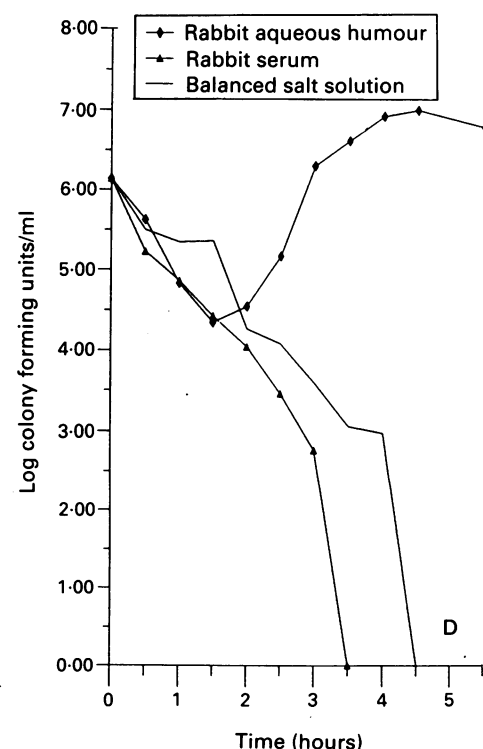
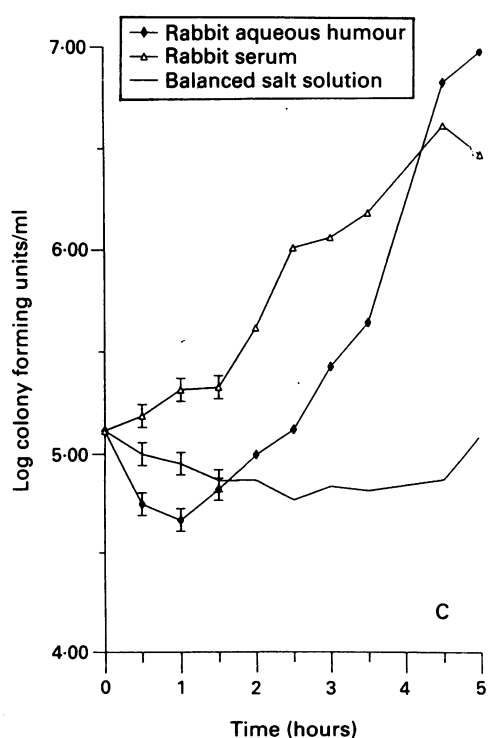
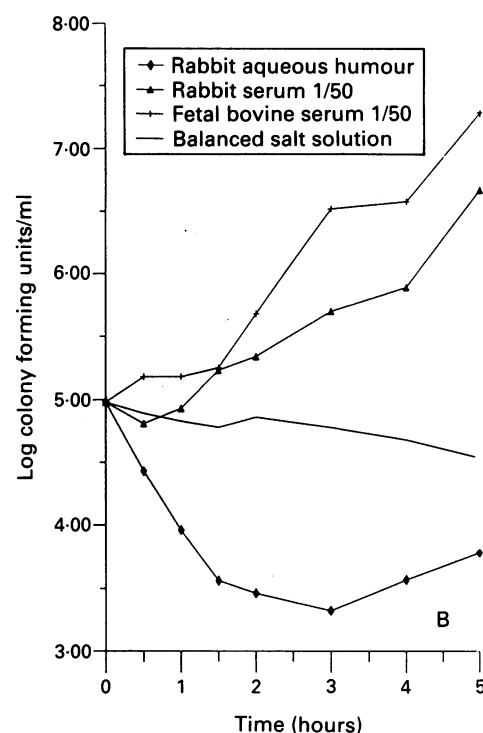
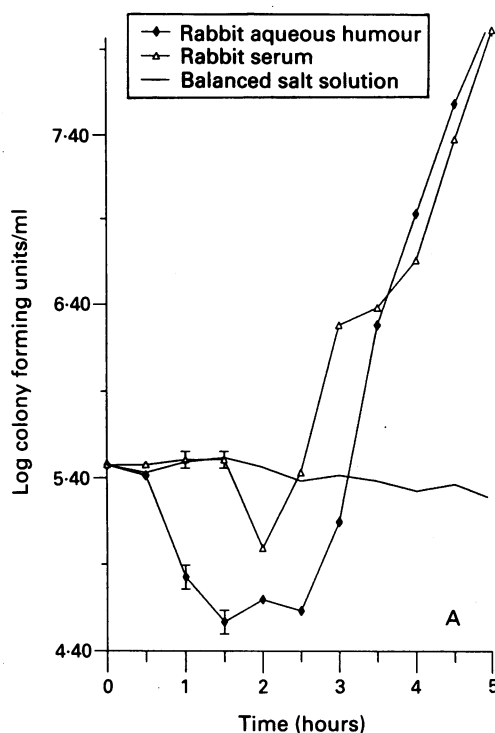
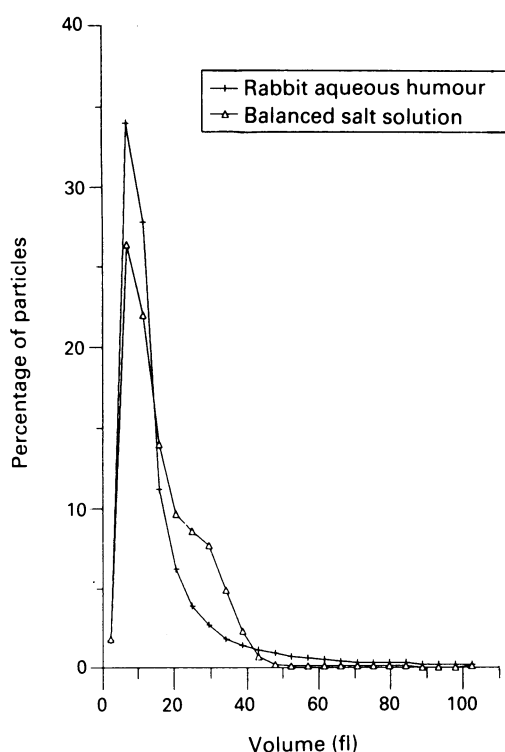


Figure 1 (A) Viability curves for *Staphylococcus aureus* NCTC No 6571 suspended in rabbit aqueous humour (RAH), rabbit serum (RS), and balanced salt solution (BSS). The difference between RAH and RS is significant between 1 and 2 hours ( $p < 0.001$ , analysis of variance, single tailed Student's *t* test,  $df=4$ ). Error bars show standard error of the mean. (B) Viability curves for *Staphylococcus aureus* NCTC No 6571 suspended in rabbit aqueous humour and 1/50 dilutions (into BSS) of rabbit serum and heat inactivated fetal bovine serum. (C) Viability curves for *Pseudomonas aeruginosa* NCTC No 10662 suspended in RAH, RS, and BSS. The difference between RAH and BSS is significant between 0.5 and 1.5 hours ( $p < 0.01$ , analysis of variance with Scheffé's method for general comparison of means). Error bars show standard error of the mean. (D) Viability curves for *Staphylococcus epidermidis* NCTC No 4276 suspended in RAH, RS, and BSS.

Figure 2 Particle (CFU) volume of *Staphylococcus aureus* NCTC No 6571 cultured for 90 minutes in rabbit aqueous humour and balanced salt solution and then fixed with 20% glutaraldehyde before measurement using a 50  $\mu$ m aperture on a Coulter counter ZM.



significant between 30 and 90 minutes ( $p < 0.01$ , analysis of variance with Scheffé's method for general comparison of means). There was no antibacterial effect mediated by aqueous humour upon *Micrococcus* spp, *Escherichia coli*, or *Streptococcus pneumoniae*. After an initial decline, *Staphylococcus epidermidis* grew better in aqueous humour than in the controls (Fig 1D).

#### CONSISTENCY OF EFFECT

Although there was some variability in potency and duration of antibacterial effect between different batches of aqueous humour, there was

minimal intrabatch variation between samples. The effect was also present in animals bred for research.

#### BACTERIAL PARTICLE SIZE

Figure 2 illustrates particle size for *Staphylococcus aureus* cultured for 90 minutes in aqueous humour and BSS. There was minimal difference between the two groups indicating that the observed reduction in colony forming units was not due to bacterial aggregation.

#### LONGEVITY OF THE BACTERICIDAL EFFECT

Figure 3A shows a repeated bactericidal effect caused by replacing the aqueous humour after 3 hours.

#### STABILITY OF THE BACTERICIDAL EFFECT

Figure 3B shows the effect of incubation at various temperatures upon aqueous humour before bacterial inoculation. Storing aqueous humour for 1 hour at temperatures between 4 and 37°C abrogated the effect. The effect was also destroyed after 30 minutes at 56°C. The effect would survive filtration through an Acrodisc 0.2  $\mu$ m low protein binding filter but was lost after passing through a Minisart cellulose acetate 0.2  $\mu$ m filter (Fig 4A and 4B). The effect survived repeat freezing in liquid nitrogen but was progressively reduced after serial dilution into BSS (Fig 4C).

The effect could be maintained over longer periods by storing the aqueous humour at 37°C in 5% CO<sub>2</sub> (Fig 4D). The difference between the killing curves for aqueous humour stored in air and 5% CO<sub>2</sub> was significant between 2 and 2.5 hours ( $p < 0.001$ , analysis of variance). A 5% CO<sub>2</sub> atmosphere acts to buffer the aqueous humour pH (Fig 5) which may be the mechanism by

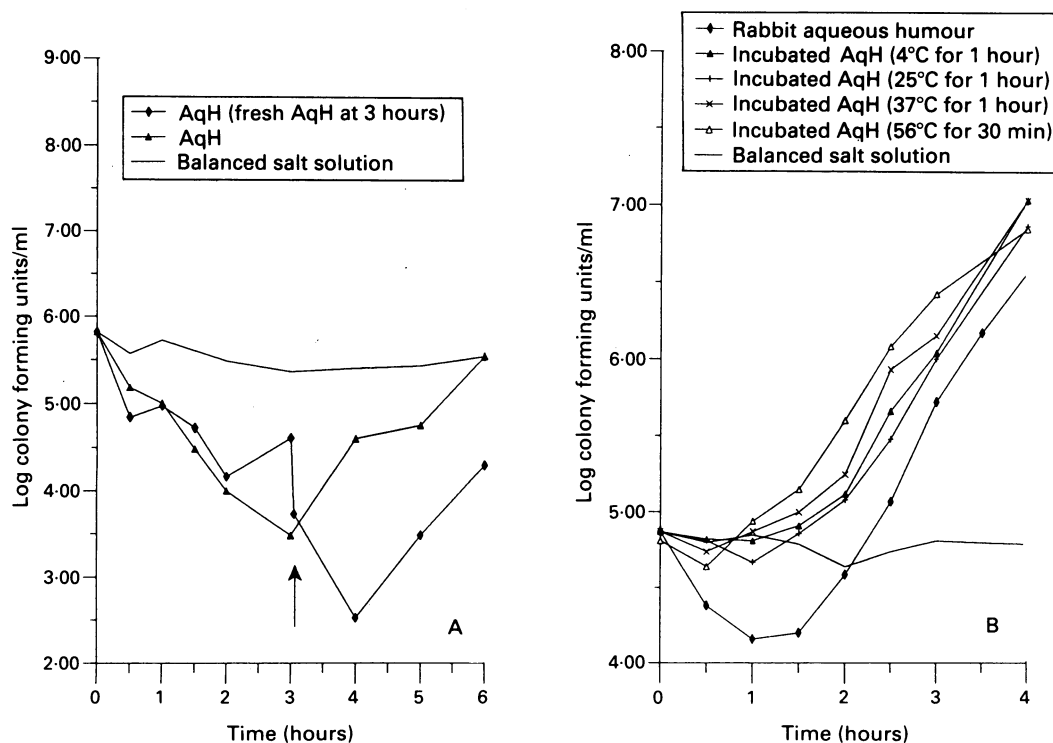


Figure 3 (A) Viability curves for *Staphylococcus aureus* NCTC No 6571 suspended in rabbit aqueous humour. After 3 hours (arrow) the suspension was spun and the organisms transferred into freshly thawed aqueous humour. (B) Viability curves for *Staphylococcus aureus* NCTC No 6571 suspended in rabbit aqueous humour (AqH) pretreated by storage at different temperatures for 30 minutes or 1 hour.

which the antibacterial effect is maintained over the longer term.

#### AQUEOUS HUMOUR ANALYSIS

There were no differences evident in SPS-PAGE profiles of samples of aqueous humour which did and did not mediate the antibacterial effect. Table 1 illustrates the electrolyte and protein content of those same samples. No appreciable differences were observed between the samples examined. There were no white blood cells identified after aqueous humour centrifugation.

#### Discussion

This investigation has demonstrated an approximate 90% drop in viable colony count for *Staphylococcus aureus* and *Pseudomonas aeruginosa* cultured for 2–3 hours in rabbit primary aqueous humour when compared with a range of controls. The controls included whole rabbit serum (containing antibacterial factors including immunoglobulin and complement), dilute rabbit serum (containing a total protein concentration closer to that of the aqueous humour), dilute heat treated fetal bovine serum (containing a similar amount of protein to

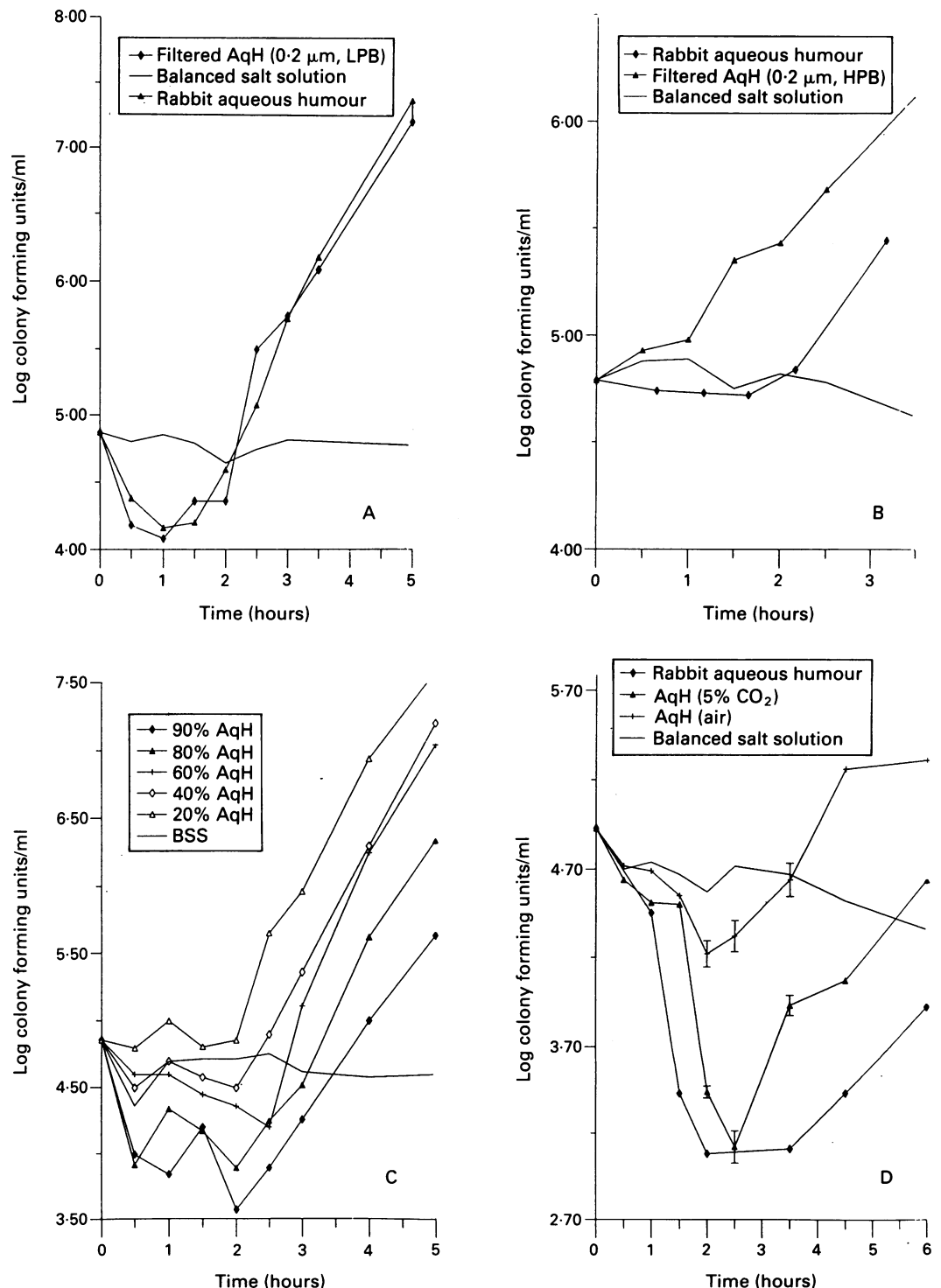
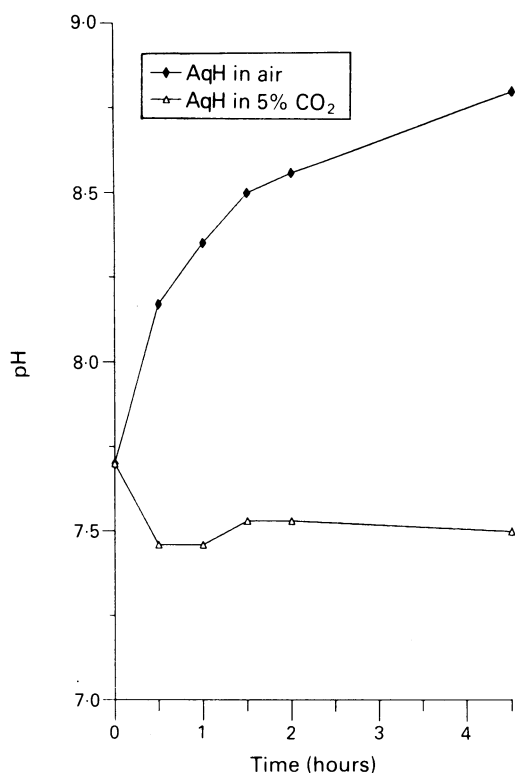


Figure 4 (A) Viability curves for *Staphylococcus aureus* NCTC No 6571 suspended in rabbit aqueous humour (AqH) filtered via a 0.2 µm low protein binding filter (LPB) (Acrodisc 0.2 µm low protein binding filter, Gelman Sciences, Ann Arbor, MI). (B) Viability curves for *Staphylococcus aureus* NCTC No 6571 suspended in rabbit aqueous humour (AqH) filtered via a 0.2 µm high protein binding (HPB) filter (0.2 µm Minisart, Sartorius GmbH, Gottingen, Germany). (C) Viability curves for *Staphylococcus aureus* NCTC No 6571 suspended in rabbit aqueous humour (AqH) pretreated by serial dilution with balanced salt solution (BSS). (D) Viability curves for *Staphylococcus aureus* NCTC No 6571 suspended in rabbit aqueous humour (AqH) pretreated by storage at 37°C for 3 hours in either air or 5% CO<sub>2</sub>. The difference between air and CO<sub>2</sub> is significant between 120 and 150 minutes ( $p < 0.0001$ , analysis of variance). Error bars show standard error of the mean.

Figure 5 pH of rabbit aqueous humour (AqH) stored at 37°C in air and 5% CO<sub>2</sub>.



aqueous humour, but minimal immunoglobulin and no complement), and balanced salt solution – an approximation to protein free aqueous humour.

The quantitative techniques applied count the number of viable colony forming units (CFU) per unit volume. These methods cannot differentiate between bacterial destruction and bacterial aggregation because clumps of bacteria give rise to single colonies. To exclude the latter as the cause for the decline in CFUs a Coulter counter was used to measure cell volume. For bacterial aggregation to explain the 90% reduction seen in bacterial units, the mean particle volume for organisms cultured in aqueous humour would have to be approximately 10 times that for the control group (BSS). There was no such difference shown, demonstrating that the reduction in CFU count may be apportioned to bacterial destruction.

Rabbit primary aqueous humour was bactericidal in vitro for *Staphylococcus aureus* and *Pseudomonas aeruginosa*. It was not bactericidal for *Micrococcus* spp, *Escherichia coli*, or *Streptococcus pneumoniae*. After an initial decline in numbers, *Staphylococcus epidermidis* grew better in aqueous humour than in the controls. It is interesting to note that *Staphylococcus epidermidis*

is the commonest cause of postoperative endophthalmitis.<sup>18</sup>

The killing curves demonstrated a drop in bacterial count extending between 2 and 3 hours following bacterial inoculation. After this period the bacterial counts recovered and entered a period of logarithmic increase. Bacterial growth recovers either because the mediator of the antibacterial effect is exhausted or because it is unstable but not because the organisms develop resistance to its effect.

There was interbatch variation in the potency of the antibacterial effect between different groups of rabbits (but minimal intrabatch variation). Different aqueous humour samples retained potency for periods of between 90 and 180 minutes after inoculation. A small number of samples did not demonstrate any bacterial killing. There was, however, no apparent difference in aqueous humour total protein content, electrolytic protein profile (SDS-PAGE), or electrolyte constitution between samples of aqueous humour which mediated and did not mediate the effect.

The total protein content of the 'primary' aqueous humour collected from farmed animals (approximately 100 mg/100 ml) is higher than the expected figure of 12.4 (SD 2.00) mg/100 ml protein.<sup>19</sup> This elevated protein level allied to the variability in the presence and potency of the antibacterial effect may reflect differences in the conditions under which these farm animals were kept before slaughter. Although it is possible that the farmed animals were given dietary supplements or antibiotics which could influence aqueous humour constitution, the bactericidal effect was also demonstrated in laboratory bred rabbits kept under controlled conditions.

The factor survived filtration through a 0.2 µm low protein binding filter but was lost after filtration through a Minisart 0.2 µm filter. It survived refreezing in liquid nitrogen but was labile when stored in air at temperatures of 4, 25, and 37°C for 1 hour. The effect was stable when the aqueous humour was stored in an atmosphere of 5% CO<sub>2</sub> at 37°C. Although storage in 5% CO<sub>2</sub> buffered the pH of the aqueous humour it is not clear whether this influence was important in maintaining the antibacterial effect.

Primary rabbit aqueous humour contains 2.0 mg/100 ml of IgG<sup>20</sup> but no IgD, IgA, or IgM.<sup>21</sup> Primary aqueous also contains complement fractions C1, C3, C4, and C5 in aqueous/serum ratios ranging from 1/1415 to 1/6713.<sup>20</sup> These levels will probably be too small to significantly affect invading bacteria. Furthermore, immunoglobulin would be expected to withstand the pretreatment regimens which removed the antibacterial effect from aqueous humour and complement should withstand 60 minutes at 4°C.

Lactoferrin and transferrin are bacteriostatic proteins which limit bacterial replication by binding available iron.<sup>22</sup> The presence of these compounds would be unlikely to explain the bactericidal effect demonstrated. Although lysozyme is bactericidal, it is more active against *Micrococcus* spp than against *Staphylococcus* spp<sup>23</sup> which is not consistent with the spectrum of activity observed in this investigation.

Table 1 Electrolytic constitution and protein content of bactericidal factor positive and negative samples of rabbit aqueous humour

Sample	1	2	3
Bactericidal	Yes	No	Yes
Lactate (mM/l)	10.5	10.3	10.0
Glucose (mg/dl)	5.3	5.3	5.2
Urea (mM/l)	<0.5	<0.5	<0.5
Na (mM/l)	147	146	143
K (mM/l)	4.9	4.9	4.7
Cl (mM/l)	105	106	103
HCO <sub>3</sub> (mM/l)	27	28	26
Total protein (mg/100 ml)	115	98	104

It is interesting to speculate on the possible nature of the factor(s) present in rabbit aqueous humour. There has been interest generated recently by the discovery that a group of small antibacterial proteins originally isolated in insects are also present in mammals.<sup>24</sup> One group, the defensins, have been isolated in humans.<sup>24</sup> It is feasible that aqueous humour contains a defensin, or defensin-like compound capable of inhibiting prokaryotic cell replication.

Aqueous humour protein has been thought to be derived from the plasma, entering the anterior chamber via selective leakage through vessels of the ciliary body and iris.<sup>25</sup> Identification of a subtype of transferrin unique to aqueous humour<sup>26</sup> and of transforming growth factor beta (TGF- $\beta$ ) production by cells in the iris and ciliary body, via local transcription of mRNA<sup>27</sup> suggests that some of the proteins in aqueous humour may be synthesised within the eye. It is conceivable that there may be iris/ciliary body production of this aqueous humour antibacterial agent.

Caution must be exercised before extrapolating these results to human ocular infection because the immunological responses of different species are frequently at variance. However, when the rabbit aqueous humour antibacterial factor has been characterised, further work can be undertaken to determine whether the same effect is present in human aqueous humour. If this were the case, it could be of great significance in the apparent resilience of the eye to infection.

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